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Engineering a fluorescence biosensor for the herbicide glyphosate

Pierre-Emmanuel Y. N'Guetta, Maggie M. Fink, and Shahir S. Rizk*

Department of Chemistry and Biochemistry, Indiana University, South Bend, IN 46615, USA

*To whom correspondence should be addressed. E-mail: srizk@iusb.edu

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Abstract

Glyphosate, the active ingredient in RoundUp, is the most widely used herbicide on the globe, and has recently been linked to an increased risk in non-Hodgkin's lymphoma in exposed individuals. Therefore, detection and monitoring of glyphosate levels in water and soil is important for public safety. Here, we describe a biosensor for glyphosate based on an engineered *Escherichia coli* phosphonate-binding protein (PhnD). Mutations in the binding pocket were introduced to convert PhnD into a glyphosate-binding protein. A fluorescence group attached near the hinge of the protein was added to monitor binding of glyphosate and to determine its concentration in unknown samples. The resulting engineered biosensor can detect glyphosate in tap water and in soil samples treated with the herbicide at submicromolar concentrations, well below the limit for drinking water in the USA. Incorporating this biosensor in a device would allow rapid and continuous monitoring of glyphosate in water and soil samples.

Key words: biosensor, conformational coupling, fluorescence, glyphosate, periplasmic-binding protein, RoundUp

Introduction

Glyphosate, a synthetic phosphonate, is the active ingredient in the herbicide RoundUp that kills weeds by blocking pathways essential to plant growth (Maeda and Dudareva, 2012). Currently, glyphosate is the most popular herbicide used globally and has been classified as a probable carcinogen (Zhang *et al*., 2019). RoundUp has made recent headlines for its widespread use on genetically modified seeds and research has linked its use t to antibiotics resistance and hormone disruption (Tarazona *et al*., 2017). It is also considered a potential environmental hazard and has been linked to an increased risk of non-Hodgkin's lymphoma (Zhang *et al*., 2019). Several states are planning to restrict its use and has already been banned in California. Due to these potential negative effects, there is a need for the development of reliable detection methods of glyphosate in soil, rivers and drinking water (Valle, 2019).

Biosensors for several molecules have previously been developed using proteins belonging to the superfamily of bacterial periplasmic-binding proteins (PBPs) (Grünewald, 2013). Members of this superfamily reside in the periplasm of Gram-negative bacteria and are used to detect and transport small molecules to the cytosol for metabolism (Quiocho and Ledvina, 1996). All PBPs share a unique mechanism of ligand binding characterized by a large conformational change associated with binding in a hinge-bending motion. By attaching a fluorescence reporter group at a specific residue near the hinge region of the protein, the transition from an open (apo) to a closed (ligand-bound) form can be monitored by observing changes in the emission properties of the fluorophore (de Lorimier *et al*., 2002). PBPs bind a variety of naturally occurring molecules such as sugars, amino acids, metals, anions, each protein binding to a specific ligand or a small number of structurally related molecules. Therefore, several PBPs have been used to develop fluorescent biosensors for a large number of naturally occurring molecules (de Lorimier *et al*., 2002).

Attempts to re-engineer the binding pockets of PBPs to expand the diversity of the ligands have been met with some success (Dwyer and Hellinga, 2004; Marvin and Hellinga, 2001). The main drawback is that often the coupling of ligand binding to the large conformational change is diminished (Allert *et al*., 2004) or completely lost (Telmer and Shilton, 2005), typically due to the large number of mutations required to drastically change the ligand-binding specificity. As a consequence, the change in fluorescence associated with ligand binding

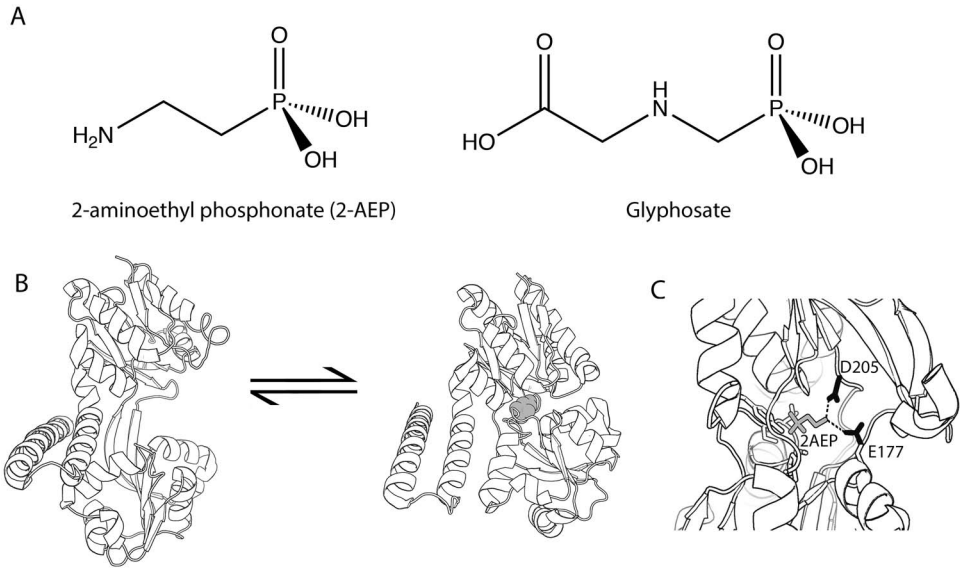

Fig. 1 (**A**) Structural similarities between glyphosate and 2-AEP, the cognate ligand for PhnD. (**B**) Ligand binding to PhnD induces a large conformational change between from an unbound open form to a ligand-bound closed form through a hinge-bending motion. (**C**) Binding pocket of PhnD showing interactions in dashed lines between 2-AEP (gray) and residues E177 and D205 (black).

is diminished, reducing their precision as biosensors. Our aim was to use phosphonate-binding protein (PhnD) as a scaffold to engineer a fluorescent biosensor for glyphosate. PhnD is a member of the PBP superfamily that binds to 2-aminoethyl phosphonate (2-AEP) (Rizk *et al*., 2006), a naturally occurring phosphonate similar in structure to glyphosate (Fig. 1A). Previous work described specific positions near the hinge of PhnD where an attached fluorophore (acrylodan) exhibits a large change in emission in response to 2-AEP binding. This method was used to determine that the protein also binds to a number of related molecules, albeit with much lower affinity (Rizk *et al*., 2006). PhnD binds to 2-AEP with a very high affinity ($K_d \sim 5$ nM), but shows low affinity for glyphosate (Alicea *et al*., 2011; Rizk *et al*., 2006). Our goal was to re-engineer the binding pocket of PhnD to accommodate glyphosate while retaining the large change in fluorescence associated with the conformational change from the open to the closed form (Fig. 1B).

Results

The crystal structure of PhnD bound to 2-AEP (Alicea *et al*., 2011) shows that the hydroxyls of residues Tyr47, Try93, Ser127, Thr128 and Ser129 coordinate the phosphonate moiety of 2-AEP, whereas the carboxylates of Glu177 and Asp205 bind to the amino group. Since 2-AEP and glyphosate share the same phosphonate moiety, we focused on replacing Glu177 and/or Asp205 to accommodate the longer glyphosate molecule and its carboxylic acid (Fig. 1C). Kunkel mutagenesis (Kunkel *et al*., 1987) was used to construct a set of single and double mutants of PhnD. The mutants also contained an additional cysteine mutation at position 125 for attachment of a thiol-reactive fluorescent reporter and a C-terminal histidine tag for purification using immobilized metal affinity chromatography.

The purified mutants were labeled with acrylodan and tested for their ability to bind to glyphosate. Dissociation constant (K_d) values were determined by monitoring the changes in fluorescence as a function of increasing ligand concentrations. Of the mutants tested, the E177N mutant (PND177N) showed the highest affinity for glyphosate with an 80-fold increase in affinity over the wild-type protein (Table I). Based on the structure of PhnD in complex with 2-AEP (Alicea *et al*., 2011), changing the glutamic acid residue to an asparagine allows for the larger glyphosate molecule to bind, while replacing a negatively charged side chain with an amide to accommodate the carboxylate of glyphosate. To further optimize the biosensor, we constructed an additional mutant (177NΔ), where the C-terminal nine amino acids were truncated. These residues have been shown to be involved in dimerization (Alicea *et al*., 2011), a property that is unique to PhnD among other PBPs. The truncated mutant showed a further improvement in the affinity for glyphosate with a K_d of 4.2 μM (Table I), enhancing the overall binding affinity by over 150-fold.

Table I. Dissociation constants (K_d values) of PhnD variants for glyphosate

PhnD variants	K_d for glyphosate
Wild type	650 μM[a]
D205N	600 μM
E177N, D205S	98 μM
E177N	8.0 μM
E177NΔ	4.2 μM

All variants contain a cysteine at position 125 for attachment of acrylodan. Values were obtained by monitoring fluorescence change as a function of ligand concentration.
[a]From Rizk *et al*., 2006

Spectral analysis of the fluorescence change associated with glyphosate binding to the sensor (PND177NΔ) showed a large decrease in fluorescence (Fig. 2A). This decrease in the fluorescence is similar to that observed with the wild-type protein (Rizk *et al*., 2006), indicating that the mutations did not severely impact the coupling of ligand binding to conformational change. Importantly, the change in fluorescence associated with ligand binding is ratiometric with a larger decrease in emission at lower wavelengths. To determine

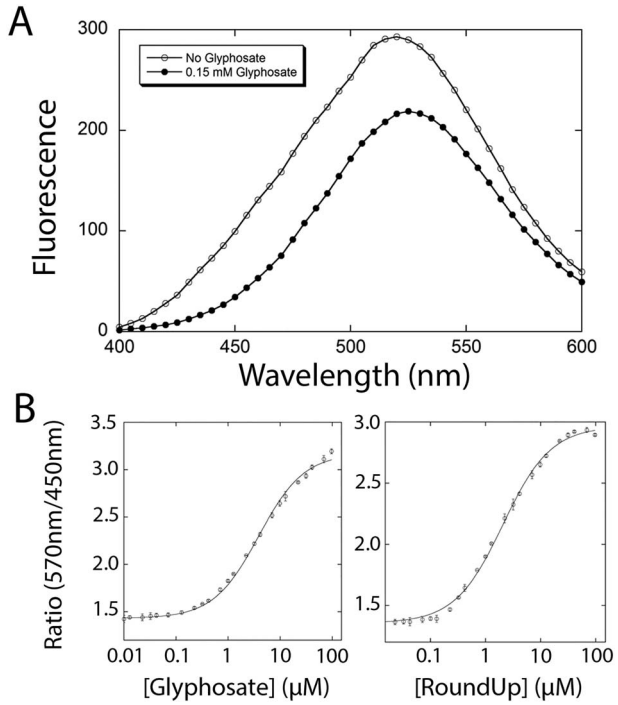

Fig. 2 (**A**) Change in fluorescence of the PhnD–E177NΔ–acrylodan conjugate in response to saturating concentrations of glyphosate. (**B**) Titration curve of PhnD–E177NΔ with glyphosate by monitoring the ratio of fluorescence at 570/450 nm. (**C**) Titration curve of PhnD–E177NΔ with RoundUp by monitoring the ratio of fluorescence at 570/450 nm.

the K_d values, the ratio of fluorescence between two wavelengths (570/450 nm) was monitored as a function of increasing ligand concentrations (Fig 2B).

We also determined the affinity of the sensor for commercial RoundUp, which contains 18.0% glyphosate isopropylamine as well as 'other ingredients'. In contrast with pure glyphosate acid, commercial RoundUp contains isopropylamine and other additives that could conceivably interfere with the ability of the sensor to detect the glyphosate content. A titration performed using diluted RoundUp solution in working buffer (50 mM 3-(N-Morpholino)propanesulfonic acid (MOPS) 150 mM NaCl, pH 6.9) revealed a $K_d \sim 2$ μM (Fig. 2C) indicating the ability of the sensor to detect the presence of glyphosate in the commercially available mixture. To examine the specificity of the sensor, we determined its affinity for a number of compounds with similar structure to glyphosate (Table II). Of all the compounds tested, the sensor exhibited the highest affinity for RoundUp, whereas the affinity for 2-AEP was greatly reduced compared with the wild-type protein by more than three orders of magnitude. The sensor also showed moderate affinities for phosphate and arsenate.

In an effort to enhance the change in fluorescence in response to ligand binding, we tested a number of thiol-reactive fluorophores. Of the fluorophores tested (see Methods section for a list of fluorophores), coumarin (7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin) showed a 6-fold decrease in fluorescence in response to RoundUp (Fig. 3A) and exhibited a similar affinity to the acrylodan conjugate with a K_d of 4 μM (Fig. 3B). To test the ability of the sensor to detect the presence of glyphosate in samples contaminated with RoundUp, several test solutions with different glyphosate concentrations were prepared by diluting

Table II. Specificity of the PhnD–E177NΔ sensor for glyphosate, RoundUp and related molecules compared with the wild-type protein (no binding pocket mutations)

Ligand	Wild-type K_d (μM)	PND177NΔ K_d (μM)
Glyphosate	650	4.29
RoundUp	ND	1.95
2-AEP	0.002	5.61
Phosphate	260	26.1
Arsenate	230	10.7

ND, no data.

RoundUp in working buffer. Initial fluorescence was recorded, then the fluorescence value was recorded again after addition of the test solution. The fluorescence values were used to calculate the concentration of each test solution. The results show a linear correlation between the expected concentrations of glyphosate and those determined using the sensor (Fig. 4A). Further, the sensor can detect concentrations as low as 1 μM, well below the permitted limit for drinking water in the USA (Noori *et al.*, 2018). We also used the sensor to test soil samples doped with RoundUp. First, RoundUp was diluted according to the manufacturer's recommendation (6 fl. oz. per gallon to a concentration of 37 mM) in either tap water or working buffer. Then the dilutions were added to soil samples obtained from the campus of Indiana University South Bend. The samples were then centrifuged, and the supernatants were further diluted to 10 μM with working buffer and added to the PND177NΔ–coumarin conjugate, and fluorescence values were used to obtain the concentration of glyphosate in each sample. The results show that the sensor can accurately detect RoundUp and determine the concentration of glyphosate in soil samples (Fig. 4B).

Discussion

Our approach in developing a biosensor for glyphosate offers several advantages. First, the biosensor described here is reagentless (de Lorimier *et al.*, 2002; Tian *et al.*, 2007), which means that no consumable reagent or a substrate is required. This is a particular advantage over current sensing methods that employ enzyme-linked immunosorbent assay or enzyme-based detection (Oliveira *et al.*, 2012). Second, no standard curve is required since the K_d value is stable over different measurements. Third, no separation, chromatography or special sample treatment is required prior to using the sensor other than addition of working buffer to maintain an appropriate pH. Furthermore, since binding of glyphosate to the protein is an equilibrium process, the sensor described here can be reused by removing the bound ligand through dialysis. Additionally, very small amounts of the protein (nanomolar concentrations) need to be used for the detection of glyphosate.

The change in fluorescence observed with the engineered sensor (PhnD177NΔ–acrylodan) resembles the overall change observed with the wild-type protein (Rizk *et al.*, 2006), showing that the mutations described here do not destroy the linkage between ligand binding and conformational change, a problem that has plagued previous work in re-engineering the binding pockets of PBPs (Allert *et al.*, 2004; Dwyer and Hellinga, 2004). Furthermore, we were able to determine that a larger change in fluorescence can be obtained by conjugating the sensor with coumarin at the same cysteine position. Using the engineered sensor described here, we are able to detect

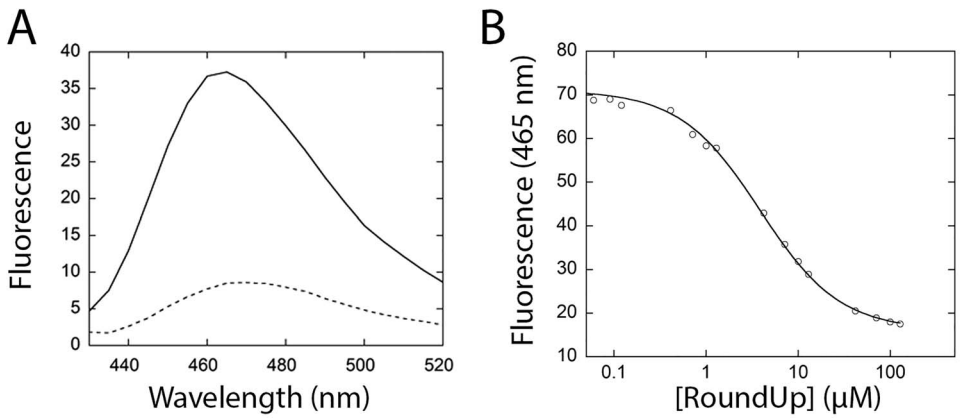

Fig. 3 (**A**) Change in fluorescence of the sensor in response to RoundUp; solid line = sensor in the absence of RoundUp, dashed line = sensor in the presence of 1 mM RoundUp. (**B**) Titration of the PND177NΔ–coumarin sensor with commercial RoundUp (K_d = 4 μM).

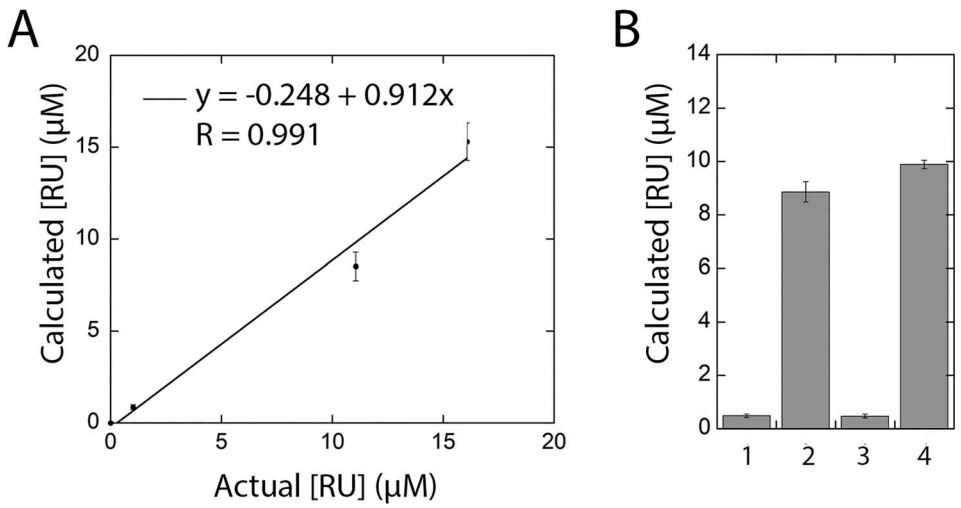

Fig. 4 (**A**) Linear correlation between actual and calculated concentrations of glyphosate in commercial RoundUp using the PND177NΔ-coumarin conjugate. (**B**) Determining concentrations of glyphosate in soil samples treated with RoundUp. Soil samples were treated with working buffer alone (1), RoundUp diluted in working buffer (2), tap water alone (3) or RoundUp diluted with tap water (4). Actual concentrations of glyphosate in 2 and 4 are 10 μM.

Roundup and accurately determine the concentration of glyphosate in soil samples.

Previous work has shown that sensors developed based on the PBP scaffold can be immobilized on a surface in a specific orientation and retain their ability to respond to the ligand (de Lorimier *et al*., 2002). As similar strategy can be used with the sensor described here to monitor glyphosate concentration in real time. This may be beneficial in water treatment facilities by monitoring pollutant concentrations in a flow cell. Fluorescence monitoring offers the ability to use mobile handheld devices for field monitoring without the use of bulky equipment. In addition, the use of PBPs for developing biosensors is not limited to fluorescence detection. Different strategies link changes in conductivity with the ligand-mediated conformational change (Tripathi *et al*., 2007), allowing for amperometric signals to indicate ligand concentration. Other strategies rely on the attachment of a redox center to PBPs to allow for an electrochemical signal change in response to ligand binding (Benson *et al*., 2001; Trammell *et al*., 2001).

The re-engineering of PhnD described here presents a great potential for its use as a sensor for the detection of glyphosate in aqueous samples. The performance of the sensor allows detection within the limit set for safe drinking water in the USA (Noori *et al*., 2018). To address potential contamination from soil phosphate, the previously reported phosphate sensor based on the bacterial phosphate-binding protein (de Lorimier *et al*., 2002) can potentially be used in a multi-sensor array to simultaneously detect both phosphate and glyphosate in the soil.

In order to detect lower concentrations of glyphosate in the soil, more mutations within the binding pocket can be explored. Alternatively, methods have been described to enhance the affinity of a PBP without making mutations within the binding pocket by either introducing bulky groups near the hinge region (Marvin and Hellinga, 2001) or by generating synthetic antigen binders that bind specifically to the closed form of the protein (Mukherjee *et al*., 2018; Rizk *et al*., 2011). Both of the strategies would have the effect of enhancing the affinity of the sensor for glyphosate, allowing detection of nanomolar amounts of the pollutant. PhnD in particular offers a unique scaffold for the design of novel biosensors owning to its natural affinity to bind to organophosphates. This group of molecules includes a wide range of environmental pollutants, pesticides,

herbicides and nerve agents like sarin. Therefore, future redesign of the binding pocket of PhnD offers the potential to generate biosensors for this array of molecules belonging to the phosphonate family.

Methods

Cloning and site-directed mutagenesis

The *phnD* gene containing the N125C mutation was cloned into a pET21a expression vector with a C-terminal 6-histidine tag as described (Rizk *et al*., 2006). Kunkel mutagenesis (Kunkel *et al*., 1987) was used to introduce point mutation within the binding pocket of PhnD (E177N, E177N/D205S, D205N, D205T) as well as a deletion of the C-terminal six amino acids responsible for dimerization (PhnDΔ and PhnD177NΔ). Sequences of all mutants were confirmed by DNA sequencing at the genomics facility at the University of Chicago.

Protein expression and purification

BL-21 DE3 chemically competent *Escherichia coli* cells were transformed by heat shock. A single colony was used to inoculate an overnight culture of 2XYT supplemented with ampicillin. The overnight culture was used to inoculate 500 ml 2XYT medium with ampicillin, and induced at OD_{600} of ∼0.8–1 with 1 mM Isopropyl β- d-1-thiogalactopyranoside (IPTG), then allowed to grow for an additional 4 h at 37°C with shaking at 300 rpm. Cells were harvested by centrifugation for 10 min at 8000 rpm, resuspended in 10 ml of 20 mM Tris, 500 mM NaCl, 10 mM Imidazole pH 8.6, sonicated on ice for 6 min with 30 s on/off intervals, then centrifuged again at 8000 rpm for 30 min to 1 h to separate the lysate from the pellet. Proteins were purified from the lysate using immobilized affinity chromatography on an ÄKTA Start system and eluted using a linear gradient from 10–300 mM imidazole. Polyacrylamide gel electrophoresis was used to verify the size of the protein, then the samples were buffer exchanged into a working buffer: 50 mM MOPS, 150 mM NaCl pH 6.9 using a desalting column (Econo-Pac DG10 from Bio-Rad). Typical yield is 2–3 mg of protein per liter of culture.

Fluorophore conjugation

The proteins were incubated with a 10-fold molar excess of fluorophore (stock concentration of 10 or 20 mM in Dimethyl sulfoxide (DMSO)) in the presence of 1 mM tris(2-carboxyethyl)phosphine (TCEP). The fluorophores tested were acrylodan (6-acryloyl-2-dimethylaminonaphthalene), IANBD (*N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-Dimethyl sulfoxidediazol-4-yl)ethylenediamine), coumarin (7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin), AlexaFluor 488 C5 maleimide, Texas Red maleimide and tetramethyl rhodamine maleimide. Reactions were incubated overnight on an inverter, then a desalting column was used to remove excess fluorophore. The labeled proteins were dialyzed three times for at least 2 h in working in 2 l of working buffer to remove any bound ligand.

Fluorescent screening and data analysis

All fluorescence measurements were collected on a SpectraM2 fluorescence spectrometer at 22°C. Acrylodan excitation was set up at 359 nm and an emission sweep was collected between 400 and 600 nm. Coumarin conjugated PhnD was excited at 419 nm and an emission spectrum was collected between 430 and 520 nm. Titrations were performed by increasing concentration of ligand and monitoring the changes in fluorescence intensity at 465 nm for coumarin conjugates or the ratio of fluorescence at 570/450 nm. Error bars represent the standard deviation from three individual titrations. The fluorescence intensity at each wavelength was measured three times for each ligand concentration and the average of the reading was calculated. The ratio of the two averages from each of the three titrations was used to calculate the standard deviation. K_d values were determined by fitting the titration data to the following single binding isotherm equation using Kaleidagraph, where F is the observed fluorescence at a certain ligand concentration [L], F_o is the fluorescence in the absence of ligand and F_{max} is the fluorescence at saturating ligand concentrations.

$$F = F_o + \frac{F_{max} - F_o}{1 + \frac{K_d}{[L]}}$$

To determine the concentration of glyphosate in standard solutions and soil extracts, the initial fluorescence, F_o, of thePND177NΔ–coumarin conjugate was recorded, then a sample of the test solution was added to the PND177N and the fluorescence (F_L) at 465 nm was recorded. Saturating amounts of glyphosate were then added to the sensor following each measurement to establish the maximum fluorescence, F_{max}. The obtained fluorescence values were used to calculate the concentration of RoundUP using the following equation:

$$[\text{RoundUp}] = \frac{K_d\,(F_L - F_o)}{F_{max} - F_o}$$

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References

Alicea, I., Marvin, J.S., Miklos, A.E. *et al.* (2011) *J. Mol. Biol.*, **414**, 356–369.
Allert, M., Rizk, S.S., Looger, L.L. *et al.* (2004) *Proc. Natl. Acad. Sci. U. S. A.*, **101**, 7907–7912.
Benson, D.E., Conrad, D.W., de Lorimier, R.M. *et al.* (2001) *Science*, **293**, 1641–1644.
de Lorimier, R.M., Smith, J.J., Dwyer, M.A. *et al.* (2002) *Protein Sci.*, **11**, 2655–2675.
Dwyer, M.A. and Hellinga, H.W. (2004) *Curr. Opin. Struct. Biol.*, **14**, 495–504.
Grünewald (2013) *Advances in Chemical Bioanalysis Bioanalytical Reviews*, F.M., M. (ed). Cham, Springer.
Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) *Methods Enzymol.*, **154**, 367–382.
Maeda, H. and Dudareva, N. (2012) *Annu. Rev. Plant Biol.*, **63**, 73–105.
Marvin, J.S. and Hellinga, H.W. (2001) *Proc. Natl. Acad. Sci. U. S. A.*, **98**, 4955–4960.
Mukherjee, S., Griffin, D.H., Horn, J.R. *et al.* (2018) *J. Biol. Chem.*, **293**, 2815–2828.
Noori, J.S., Dimaki, M., Mortensen, J. and Svendsen, W.E. (2018) *Sensors (Basel)*, **18**. doi: 10.3390/s18092961.

Oliveira, G.C., Moccelini, S.K., Castilho, M. *et al*. (2012) *Talanta*, **98**, 130–136.
Quiocho, F.A. and Ledvina, P.S. (1996) *Mol. Microbiol*., **20**, 17–25.
Rizk, S.S., Cuneo, M.J. and Hellinga, H.W. (2006) *Protein Sci*., **15**, 1745–1751.
Rizk, S.S., Paduch, M., Heithaus, J.H. *et al*. (2011) *Nat. Struct. Mol. Biol*., **18**, 437–442.
Tarazona, J.V., Court-Marques, D., Tiramani, M. *et al*. (2017) *Arch. Toxicol*., **91**, 2723–2743.
Telmer, P.G. and Shilton, B.H. (2005) *J. Mol. Biol*., **354**, 829–840.
Tian, Y., Cuneo, M.J., Changela, A. *et al*. (2007) *Protein Sci*., **16**, 2240–2250.
Trammell, S.A., Goldston, H.M.Jr., Tran, P.T. *et al*. (2001) *Bioconjugate Chem*., **12**, 643–647.
Tripathi, A., Wang, J., Luck, L.A. and Suni, I.I. (2007) *Anal. Chem*., **79**, 1266–1270.
Valle, A.L.M., C, F.C., Alves-Balvedi, R.P. *et al*. (2019) *Environ. Chem. Lett*., **17**, 291–317.
Zhang, L., Rana, I., Shaffer, R.M. *et al*. (2019) *Mutat. Res*., **781**, 186–206.